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Identification of a novel splice variant of the haloacid dehalogenase - PHOSPHO1

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Summary

PHOSPHO1, a new member of the haloacid dehalogenase superfamily, has recently been implicated in the mineralization process in both osteoblasts and chondrocytes. In this study we describe the identification of a novel, alternatively spliced PHOSPHO1 transcript (PHOSPHO1-3a). This transcript contains the 3 exons of the previously published variant, however exon 3 contains a retained, 127bp section of intron 2. This forms an in-frame start site, producing an open reading frame of 879bp and predicting a protein of 292 amino acids. The novel 40 amino acid N-terminal region of PHOSPHO1-3a contains a relatively strong secretory signal, however all three domains of the HAD superfamily are retained in exon 3. The expression of this splice variant was confirmed in both human and mouse osteoblast-like cells and also in the chondrogenic ATDC5 cell line. The data within this study indicates a possible function relating to chondrocyte differentiation/mineralization as with the previously published variant.

Introduction

PHOSPHO1 is a phosphatase enzyme belonging to the HAD superfamily of hydrolases, which has been implicated in the generation of phosphate (Pi) for skeletal mineralization [1]. Initially *Phospho1* gene studies were done in the chicken (Gallus gallus), however since then EST sequence database entries have been identified corresponding to human and murine *Phospho1* which have been subsequently amplified and cloned [2]. These transcripts have a 94% identity to each other and are 62% identical to chick *Phospho1*. The human and murine *Phospho1* genes lie within approximately 7.4Kb regions on HSA17q21.32 and distal MMU11 respectively, are flanked by the ACLY and GH1 loci, and exhibit conservation of synteny with part of GGA27. Using genetic linkage analysis, chick *Phospho1* was mapped to the syntenic region on GGA27 [2]. This strongly suggests that the *Phospho1* loci on HSA17 and MMU11 are orthologous to the *Phospho1* gene originally identified in the chick. *Phospho1* has since been identified in a number of species including rat, zebrafish and puffer fish through EST analysis [3].

PHOSPHO1 has been shown to have a high specific phosphohydrolase activity toward phosphoethanolamine (PEA) and phosphocholine (PCho) substrates [4]. PEA and PCho are involved in the biosynthesis of phosphatidylethanolamine and phosphatidylcholine, respectively, and form the polar headgroups of phospholipids in biological membranes. Both PEA and PCho levels are controlled *in vivo* by the expression of CTP-dependent cytidyltransferases, which catalyse the formation of CDP-ethanolamine and CDP-choline, respectively [5; 6].

Phospho1 is expressed at high levels in bone tissue when compared with non-skeletal tissues in both chick and murine models [7; 8]. Immunolocalization studies have shown that PHOSPHO1 is specifically localized to mineralizing regions of skeletal

tissue in both species [9]. In addition to the protein localization studies, *Phospho1* transcript has also been localized in developing embryonic bones of the chick by in situ hybridization [1]. In this study the transcript of *Phospho1* has been found to be expressed at sites destined for mineralization. Interestingly the transcript is present at these sites prior to overt signs of mineralization thereby providing support to the suggestion that PHOSPHO1 is involved in the initial stages of mineralization.

This expression/localization pattern suggests PHOSPHO1 is closely associated with the mineralization process and in particular the generation of Pi for hydroxyapatite production. This is an essential phase of mineral formation, not only for the precipitation with calcium but initially to raise the Pi/PPi ratio to a degree that is permissive to mineralization. This is due to PPi acting as a powerful inhibitor of HA formation [10; 11]. In addition, reduction of PHOSPHO1 activity through the use of newly discovered small molecule inhibitors causes a decrease in the capacity of MVs to calcify *in vitro*. These data indicate that PHOSPHO1 has a role in the mineralization process which is likely to be in the production of Pi for calcium phosphate precipitation.

In this study we now describe the discovery of a novel *Phospho1* transcript which is expressed in human osteoblasts. In addition, we present data to suggest that both transcripts exist in murine osteoblasts and chondrocytes, suggesting a role in the differentiation/mineralization of these skeletal cells.

Materials and Methods

PCR Amplification of *Phospho1* variants

Total RNA was isolated from SaOS-2 human osteoblast-like cells by phenol/chloroform extraction and treated with DNase I (Ambion) according to the manufacturer's instructions. The RT-PCR reaction was carried out using the SUPERSCRIPT - First Strand synthesis system for RT-PCR with Oligo dT (Roche). Primer pairs for β-Actin RNA were used as a control. The reaction mixture contained 5μg RNA, 500ng Oligo dT, 2μl 10 x RT buffer, 2mM $MgCl_2$, 10mM DTT, 0.5mM DNTP's, 200ul Superscript enzyme. The reaction was then cycled as follows; 25°C for 10 min, 42°C for 50 min and 70°C for 15 min. PCR primers (Exon1F 5'-CCCCCTTCCCCACTTCTTAC-3', Exon1-2-3aR 5'-CCGGGAGAGGCTGGTTAG-3'; amplicon size 230bp and Exon1F2 5'-TACCTCAGCTAGCCCCCTTC-3', Exon1-2-3bR 5'-CGGAGATGAGAATCACCTCG-3'; amplicon size 638bp and 511bp for *Phospho1*-3a and the previously published isoform respectively) were designed to allow amplification of exons created through alternative splice patterns (defined by EST analysis). Each transcript was amplified utilizing a 55°C annealing temperature and 200ng cDNA. The PCR products were then electrophoresed on a 2% agarose gel and purified using the Qiagen QiaQuick gel extraction kit. The PCR products were cloned directly into the pGEM-T-Easy vector. This plasmid is pre-lineralized and can ligate to the deoxyadenosine (A) at the 3′ ends of PCR products. The PCR product and plasmid were ligated in a 3:1 ratio using 100ng of PCR product. This mixture was used to transform JM109 competent cells. Liquid cultures and plasmid minipreparations of individual clones were subsequently carried out. DNA sequencing of plasmids was carried out by the DNA sequencing facility at Dundee University.

Cell Culture

The ATDC5 chondrocyte cell line was obtained from the RIKEN cell bank (Ibaraki, Japan), and cells cultured at a density of 6000 cells/cm^2 in differentiation medium (DMEM:F12 (Invitrogen, Paisley, UK), 5% FCS (Invitrogen), $3x10^{-8}$M sodium selenite, 10μg/ml human transferrin (Sigma, Poole, UK) and 10μg/ml insulin (Sigma)). Cells were differentiated at 37°C in a humidified atmosphere containing 5% CO_2 for up to 15 days. We and others have previously shown that from 8 days of differentiation, ATDC5 cells express the chondrocyte marker gene collagen type II, and by day 15, the cells display a terminally differentiating phenotype, with increased levels of collagen type X and aggrecan expression, and the formation of nodules [12; 13]. Some cultures were further differentiated for 28 days to induce mineralization in the presence of 10mM β-glycerol phosphate (βGP) and 50 μg/ml ascorbate-2-phosphate.

Mouse calvarial cells (wild-type Ob) were isolated from 3-day-old mice through sequential collagenase digestion. Calvarial cells were plated at a density of 20,000/cm^2 in alpha-MEM (Gibco, Paisley, UK) containing 10% FBS. MLO-A5 [14] cells were obtained from L.F. Bonewald (University of Missouri, Kansas City, MO) and cultured in a similar manner but seeded at 10,000/ cm^2.

PCR of Genes During ATDC5 Differentiation

RNA was extracted and reverse transcribed as described above. Primers for 18s RNA were purchased from Applied Biosystems (Warrington, UK). Other primers were designed using the software programme Primer3 (Whitehead Institute for Biomedical Research); *Phospho1:* forward 5′- GACAATGAGCGGGTGTTTTC-3′, reverse 5′-GGGGATGGTCTCGTAGACAG-3′; *Aggrecan*: forward 5′-

CGAGAATGACACCTGCTAGG-3 reverse 5′-AAGAAGACAGGACCAGGAAGG-3', *Collagen II* (*Coll2a*): forward 5′-GCCAAGACCTGAAACTCTGC-3 reverse 5′-GGTTGGGGTAGACGCAAGTC-3'. The PCR reaction contained; 0.2mM dNTP mix (Promega), 5μl 10x PCR Buffer (Roche), 5 units Taq polymerase (Roche), 0.5μM of the forward and reverse primers and 1μl cDNA. This was then cycled at: 94°C for 5 minutes, thirty cycles of 94°C for 30 seconds 55°C for 30 seconds and 72°C for 1 minute and finally one step on 72°C for 10 minutes. Each reaction was analyzed on 1.5% agarose gels run in the presence of ethidium bromide (250 μg/l).

Western Blotting

Cell extracts were analyzed for the presence of PHOSPHO1 by immunoblotting. Cell preparations were lysed in PBS containing 1.6 mg/ml of Complete® protease inhibitor cocktail (Roche, Lewes, UK). Samples corresponding to 50 μg total protein were incubated at 70˚C for 10 minutes in LDS sample buffer before loading. Samples were run on a 10% Bis-Tris NuPAGE gel and electroblotted to nitrocellulose, which were incubated in blocking solution (5% non-fat milk in Tris buffered saline with 0.1% Tween 20). The membranes were then probed with a 1:750 dilution of rabbit-anti-PHOSPHO1 antisera in blocking solution and washed three times with PBS. Blots were then incubated with goat anti rabbit IgG-peroxidase (DAKO) diluted 1:2,000 in blocking solution. The immune complexes were then visualized by enhanced chemiluminescence

Results

Identification of a novel splice variant of *Phospho1*

We have previously reported the high expression of *Phospho1* in both mineralizing growth plate chondrocytes and osteoblasts from murine sources. During these previous studies [8] a doublet band was observed upon amplification of *Phospho1* between exons 2 and 3 and we have previously postulated that this was an alternative transcript from the *Phospho1* gene. The presence of this doublet was confirmed in both primary osteoblasts and MLO-A5 cells in this present study (Figure1A). A search of ESTs from the human NCBI database revealed a sequence assigned **BC029931** that perhaps reinforced this hypothesis. This sequence from the *Phospho1* gene had a section of retained intron from intron 2 (Figure 2). In order to investigate the possibility of an alternative splice variant, primers were designed to amplify a product containing exon one to three. Primers were designed to simultaneously amplify both the variant containing the novel exon – termed exon 3a, and the original isoform. The amplification products were cloned and sequenced. As seen in Figure 1B both splice variants can be amplified from SaOS-2 osteosarcoma cDNA, each identical apart from a 127bp exon found at the 5' region of exon 3 (Figure 2B). The novel transcript can be amplified both independently (Figure 2B lane 2) and along with the previously published transcript (Figure 2B lane 3), depending on the position of the reverse primer. This novel variant is termed *Phospho1*-3a. This novel exon causes a frame shift, which in turn causes the initiation site from exon 2 to be lost and a novel start site to be created in exon 3a. Consequently, an open reading frame of 879 nucleotides is formed, which can be translated into a protein of 292 amino acids. All three motifs which form the conserved active site from the HAD superfamily are conserved between both splice variants as they are all located within exon 3.

Expression of *Phospho1*-3a in differentiating chondrocytes

The *Phospho1* splice variants were amplified using primers which crossed intron 2 and exon 3a of the *Phospho1* gene, in order that both variants would be amplified together. As can be seen in figure 3A, the ATDC5 cell line progresses from a proliferating phenotype at day 0 to a terminally differentiated phenotype with the presence of cartilaginous nodules (as indicated by arrows) at day 15. Both splice variants are identified in the differentiating cells with apparent equal intensity, however there is an increase in both during the initial stages of differentiation, between days 0 and 4 (Figure 3B). The differentiation of the chondrocytes is confirmed by the increase in expression of *Aggrecan* during the differentiation process. The presence of *Collagen II* (*Coll2a*) transcript verifies the chondrocyte like phenotype of the cells.

PHOSPHO1 Protein Levels in Differentiating Cells

To analyze the effect of differentiating factors on the presence of PHOSPHO1 within mineralizing ATDC5 cells, the cells were cultured in the presence of 10 mM βGP and 50 μg/ml ascorbate-2-phosphate. Two forms of PHOSPHO1 (32 and 29KDa) were detected in each cell type corresponding to two splice variants from the *Phospho1* gene, as seen in figure 3C. However, PHOSPHO1 appeared to be constitutively expressed and protein levels did not alter during the latter phase of matrix mineralization. Mineralization was evident by day 22 (data not shown).

Discussion

In this study, we identified a novel splice variant from the *Phospho1* gene, named *Phospho1*-3a. The analysis of this splice variant reveals a protein with an alternative N terminal region (Figure 4), termed exon 3a. This exon is formed from a retained 127bp section of intron 2, leading to the formation of a new in frame ATG initiation site giving rise to a novel 334bp ORF.

This is not uncommon within the haloacid dehalogenase superfamily, in fact H^+-K^+-ATPases show a somewhat similar splicing pattern with alternative transcriptional initiation and mRNA splicing giving rise to distinct N-terminal variants of the HKalpha2 subunit [15]. The splice variant of *Phospho1* only differs in the N terminal region, thus these variants are perhaps encoding PHOSPHO1 proteins with slightly different functions within or outside the cell. Interestingly, analysis of the 3a sequences with the program SignalP v3.0 [16] suggests the presence of a relatively strong secretory domain hinting that this form is either being secreted or signaled to alternative sites within the cell (data not shown). All three domains within the *Phospho1* gene are conserved within exon 3b (Figure 2a), indicating that these splice variants are functioning against a common substrate or substrate structure, as is seen in H^+-K^+-ATPases, where N-terminal alteration causes a difference in pharmacological profile but not in substrate specificity. The splicing of pre-mRNA is tightly controlled by spliceosomes following gene transcription but before translation. These spliceosomes recognise the boundary found between introns and exons, upon detection they catalyze the cutting out of intronic sequence and the joining of exons. The recognition of specific splice sites relies on the presence of conserved intronic di-nucleotides at the 5' (donor) and 3' (acceptor) exon-intron boundaries. However further sequences around the splice site are required for proper splicing [17].

PHOSPHO1 has been shown to be involved in the mineralization process through the production of Pi for hydroxyapatite crystal formation. Its expression is restricted to cells of the skeleton where it has been shown to be highly expressed by both mineralizing chondrocytes and osteoblasts [7; 8; 9]. Within the epiphyseal growth plate, PHOSPHO1 protein has been located to prehypertrophic chondrocytes and absent from both proliferating and hypertrophic regions [8; 9]. This observation suggests that PHOSPHO1 has a role in the early stages of matrix mineralization. To analyse whether *Phospho1*-3a is differentially expressed during these stages the murine chondrogenic ATDC5 cell line was induced to differentiate from a proliferating to a terminally differentiated phenotype. Both *Phospho1* transcripts were identified during this process at an apparent equal expression; however a modest increase in expression levels was noted over time, which is in agreement with Chen et al (2005) [18]. This is similar to another haloacid dehalogenase – like enzyme, Sarco/endoplasmic reticulum-type calcium transport ATPase 2, where a novel splice variant was identified to be involved in monocyte differentiation [19]. This splice variant, which is formed in a similar fashion to *Phospho1*-3a – through an inclusion of a small section of intronic sequence at the 3' region of the transcript, is upregulated during differentiation. As with PHOSHO1-3a the novel variant of SERCA2 is modulated in a similar fashion as the major isoform. This suggests that the novel splice variant from the *Phospho1* gene may be involved in chondrogenic differentiation from a proliferating to hypertrophic phenotype along with the previously published isoform.

As *Phospho1* has been implicated in the mineralization process, ATDC5 cells were induced to mineralize through the addition of βGP and ascorbate-2-phosphate for a period of 28 days. However, upon analysis of PHOSPHO1 protein levels directly pre

and proceeding (detectable) matrix mineralization, the transcripts appear to be constitutively expressed, although the larger, novel *Phospho1*-3a is expressed at a higher level than the previously published variant, suggesting a possible synergy between the two isoforms. Similarly, the HAD enzyme epoxide hydrolase-2 has three published isoforms which differ in their N terminal regions only [20; 21]. All three isoforms are closely linked to the ovulation process, however exact functions for these variants have yet to be identified. It is therefore possible that this is also the case with variants of *Phospho1* and the mineralization process.

Phospho1 expression has been documented in alternative tissues such as heart and bone marrow, although at considerably lower levels [8]. The identification of the splice variant *Phospho1*-3a may suggest alternative functions in tissues other than bone. Interestingly, PHOSPHO1 has also been shown to be able to cleave PCho, the hydrolysis of which has been studied in hamster heart and has been shown to be catalysed by an unidentified enzyme [22].

Recently PHOSPHO1 has been directly implicated in phase one of matrix vesicle mineralization [8]. This process involves the intra-vesicular formation of hydroxyapatite crystal and is critical for normal bone growth and maintenance. The identification of a novel splice variant from this gene may serve to provide further insights into this process or perhaps provide a means for Pi or PEA/Cho production in distinct sites. However, elucidation of the precise function and location of *Phospho1*-3a would first need to be established.

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References

[1] A.J. Stewart, S.J. Roberts, E. Seawright, M.G. Davey, R.H. Fleming, and C. Farquharson, The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization. Bone 39 (2006) 1000-7.

[2] B. Houston, I.R. Paton, D.W. Burt, and C. Farquharson, Chromosomal localization of the chicken and mammalian orthologues of the orphan phosphatase PHOSPHO1 gene. Anim Genet 33 (2002) 451-4.

[3] A.J. Stewart, R. Schmid, C.A. Blindauer, S.J. Paisey, and C. Farquharson, Comparative modelling of human PHOSPHO1 reveals a new group of phosphatases within the haloacid dehalogenase superfamily. Protein Eng 16 (2003) 889-95.

[4] S.J. Roberts, A.J. Stewart, P.J. Sadler, and C. Farquharson, Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities. Biochem J 382 (2004) 59-65.

[5] O.B. Bleijerveld, W. Klein, A.B. Vaandrager, J.B. Helms, and M. Houweling, Control of the CDPethanolamine pathway in mammalian cells: effect of CTP:phosphoethanolamine cytidylyltransferase overexpression and the amount of intracellular diacylglycerol. Biochem J 379 (2004) 711-9.

[6] C. Kent, CTP:phosphocholine cytidylyltransferase. Biochim Biophys Acta 1348 (1997) 79-90.

[7] B. Houston, E. Seawright, D. Jefferies, E. Hoogland, D. Lester, C. Whitehead, and C. Farquharson, Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes. Biochim Biophys Acta 1448 (1999) 500-6.

[8] S. Roberts, S. Narisawa, D. Harmey, J.L. Millan, and C. Farquharson, Functional involvement of PHOSPHO1 in matrix vesicle-mediated skeletal mineralization. J Bone Miner Res 22 (2007) 617-27.

[9] B. Houston, A.J. Stewart, and C. Farquharson, PHOSPHO1-A novel phosphatase specifically expressed at sites of mineralisation in bone and cartilage. Bone 34 (2004) 629-37.

[10] N.M. Hansen, R. Felix, S. Bisaz, and H. Fleisch, Aggregation of hydroxyapatite crystals. Biochim Biophys Acta 451 (1976) 549-59.

[11] T.C. Register, and R.E. Wuthier, Effect of pyrophosphate and two diphosphonates on 45Ca and 32Pi uptake and mineralization by matrix vesicle-enriched fractions and by hydroxyapatite. Bone 6 (1985) 307-12.

[12] H. Enomoto, M. Enomoto-Iwamoto, M. Iwamoto, S. Nomura, M. Himeno, Y. Kitamura, T. Kishimoto, and T. Komori, Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 275 (2000) 8695-702.

[13] T. Mushtaq, C. Farquharson, E. Seawright, and S.F. Ahmed, Glucocorticoid effects on chondrogenesis, differentiation and apoptosis in the murine ATDC5 chondrocyte cell line. J Endocrinol 175 (2002) 705-13.

[14] Y. Kato, A. Boskey, L. Spevak, M. Dallas, M. Hori, and L.F. Bonewald, Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. J Bone Miner Res 16 (2001) 1622-33.

[15] B.C. Kone, and S.C. Higham, A novel N-terminal splice variant of the rat H+-K+-ATPase alpha2 subunit. Cloning, functional expression, and renal adaptive response to chronic hypokalemia. J Biol Chem 273 (1998) 2543-52.

[16] J.D. Bendtsen, H. Nielsen, G. von Heijne, and S. Brunak, Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340 (2004) 783-95.

[17] B.M. Brinkman, Splice variants as cancer biomarkers. Clin Biochem 37 (2004) 584-94.

[18] L. Chen, T. Fink, X.Y. Zhang, P. Ebbesen, and V. Zachar, Quantitative transcriptional profiling of ATDC5 mouse progenitor cells during chondrogenesis. Differentiation 73 (2005) 350-63.

[19] P. Gelebart, V. Martin, J. Enouf, and B. Papp, Identification of a new SERCA2 splice variant regulated during monocytic differentiation. Biochem Biophys Res Commun 303 (2003) 676-84.

[20] J.D. Hennebold, K. Mah, W. Perez, J.E. Vance, R.L. Stouffer, C. Morisseau, B.D. Hammock, and E.Y. Adashi, Identification and characterization of an ovary-selective isoform of epoxide hydrolase. Biol Reprod 72 (2005) 968-75.

[21] K. Shkolnik, S. Ben-Dor, D. Galiani, A. Hourvitz, and N. Dekel, A novel ovary-specific and ovulation-associated variant of epoxide hydrolase 2. FEBS Lett 581 (2007) 4891-8.

[22] G.M. Hatch, and P.C. Choy, Phosphocholine phosphatase and alkaline phosphatase are different enzymes in hamster heart. Lipids 22 (1987) 672-6.

Figure Legends

Figure 1 PCR products corresponding to splice variants of human *Phospho1*. (A) Amplification of *Phospho1* from exon 2 to exon 3 in wild type murine osteoblasts (Ob) and pre-osteocytes (MLO-A5) and (B) amplification from exon 2 to exon 3a (lane 2) and exon 3 (lane 3) in human SaOS-2 cells. RNA was extracted from each cell type and reverse transcribed before being amplified using gene specific primers as described in "Materials and Methods".

Figure 2 Structure of a novel alternatively spliced exon 3a. (A) Schematic representation of exon organization and protein domains in the *Phospho1* splice variant. Arrows indicate alternative ATG start sites. Note the presence of exon 3a does not affect any of the HAD conserved domains located in Exon 3. (B) *Phospho1*-3a retains a 127bp portion of intron 2 (corresponding to nucleotides 44657239–44657366 of human chromosome 17) as a novel exon, termed exon 3a. *consensus sequence for RNA splicing acceptor site # consensus sequence for RNA splicing donor site.

Figure 3 The effect of chondrocyte differentiation on *Phospho1* expression. (A) The chondrogenic ATDC5 cells display phenotypic changes when differentiated in the presence of sodium selenite, transferrin and insulin, depicted by the presence of cartilaginous nodules (indicated by arrows) at day 15 of culture. (B) Expression of *Phospho1*, *Aggrecan* and *Coll2a* in ATDC5 cells during differentiation. Differentiation was confirmed by the increase in aggrecan expression. RNA was extracted from each cell type and reverse transcribed before being amplified using gene specific primers as described in "Materials and Methods". (C) Immunoblot examining PHOSPHO1 expression in protein isolated from ATDC5 cells induced to

mineralize. The cells were cultured in the presence of 10mM βGP and 50 μg/ml ascorbate-2-phosphate. Mineralization of the monolayer was evident by day 22.

Figure 4 Comparison of amino acid alignment of human regular PHOSPHO1 and PHOSPHO1-3a. The predicted amino acid sequence of PHOSPHO1-3a is aligned with the amino acid sequence of human PHOSPHO1. Note the identical sequence outwith the N-terminal region.

Figure 1

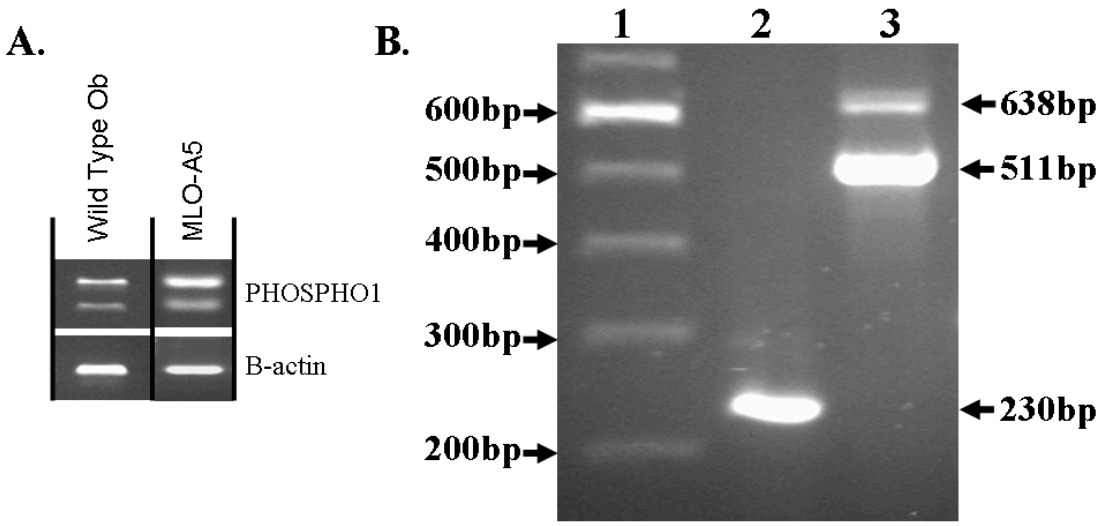
A.
Wild Type Ob
MLO-A5
PHOSPHO1
B-actin
B.
1
2
3
600bp
500bp
400bp
300bp
200bp
638bp
511bp
230bp

Figure 2

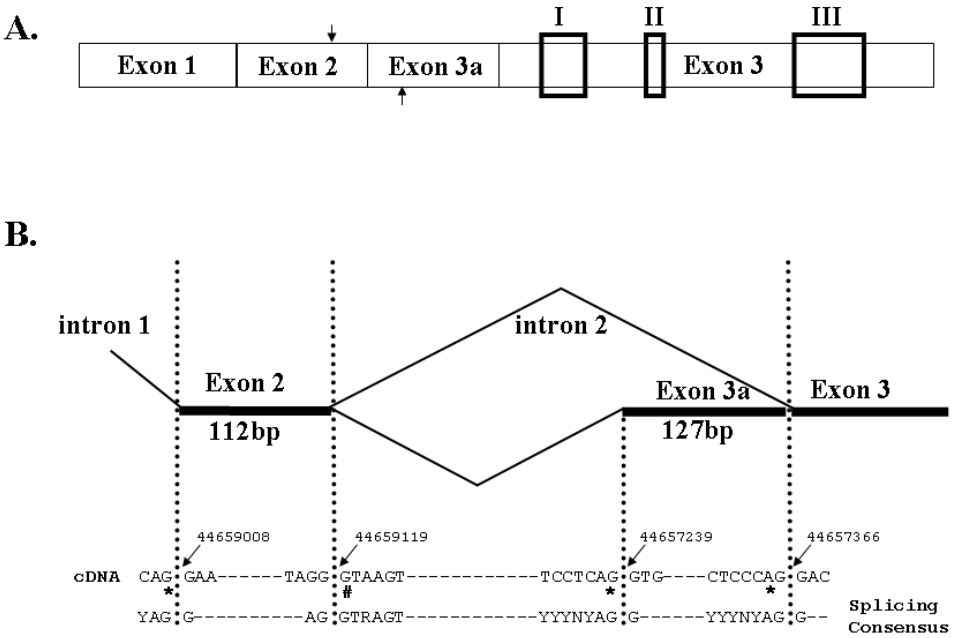
A.
Exon 1
Exon 2
Exon 3a
I
II
III
Exon 3
B.
intron 1
Exon 2
112bp
intron 2
Exon 3a
127bp
Exon 3
44659008
44659119
44657239
44657366
cDNA CAG GAA------TAGG GTAAGT----------TCCTCAG GTG----CTCCCAG GAC
YAG G----------AG GTRAGT----------YYYNYAG G------YYYNYAG G--
Splicing
Consensus

Figure 3

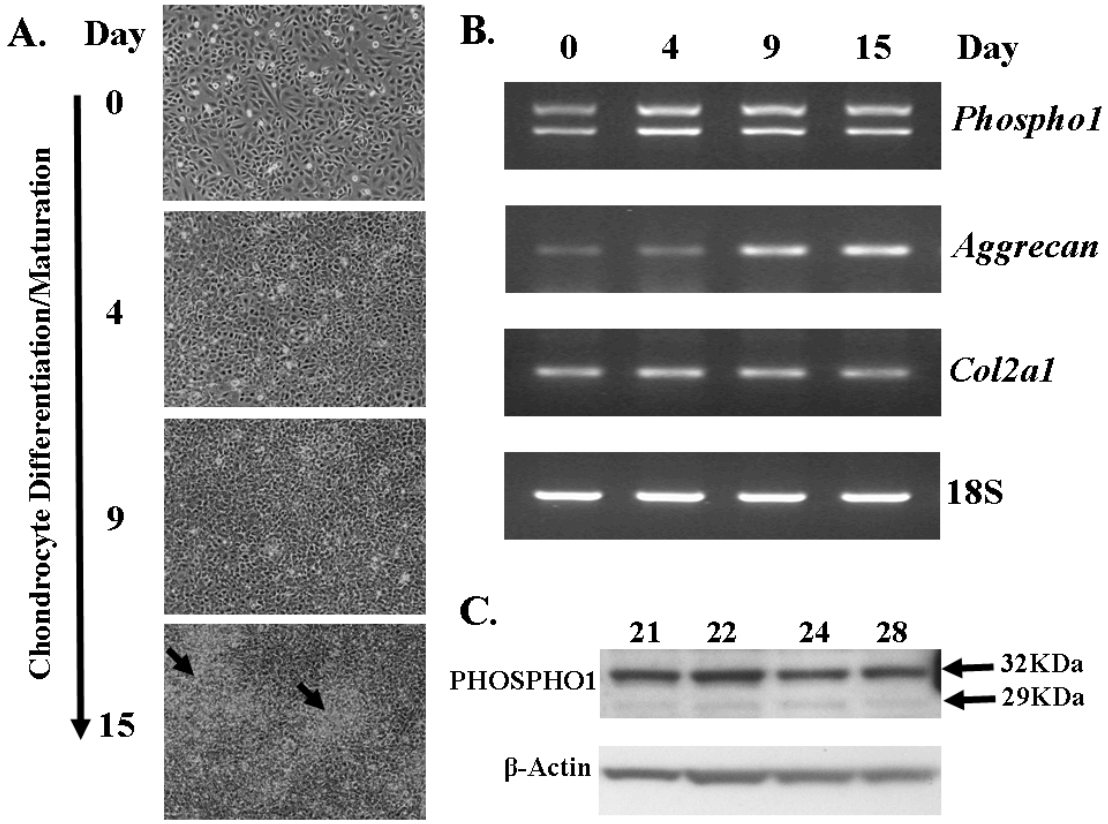
A.
Day
0
4
9
15
Chondrocyte Differentiation/Maturation
B.
0
4
9
15
Day
Phospho1
Aggrecan
Col2a1
18S
C.
21
22
24
28
PHOSPHO1
32KDa
29KDa
β-Actin

Figure 4


PHOSPHO1 ----------MSG--------CFPVSGLRCLSR----DGRMAAQGAPRFLLTFDFDE
PHOSPHO1-3a MCQRLWPWPANQPLPGGLLPRPLSLAPSSSSSCCSPPCSQDGRMAAQGAPRFLLTFDFDE
PHOSPHO1 TIVDENSDDSIVRAAPGQRLPESLRATYREGFYNEYMQRVFKYLGEQGVRPRDLSAIYEA
PHOSPHO1-3a TIVDENSDDSIVRAAPGQRLPESLRATYREGFYNEYMQRVFKYLGEQGVRPRDLSAIYEA
PHOSPHO1 IPLSPGMSDLLQFVAKQGACFEVILISDANTFGVESSLRAAGHHSLFRRILSNPSGPDAR
PHOSPHO1-3a IPLSPGMSDLLQFVAKQGACFEVILISDANTFGVESSLRAAGHHSLFRRILSNPSGPDAR
PHOSPHO1 GLLALRPFHTHSCARCPANMCKHKVLSDYLRERAHDGVHFERLFYVGDGANDFCPMGLLA
PHOSPHO1-3a GLLALRPFHTHSCARCPANMCKHKVLSDYLRERAHDGVHFERLFYVGDGANDFCPMGLLA
PHOSPHO1 GGDVAFPRRGYPMHRLIQEAQKAEPSSFRASVVPWETAADVRLHLQQVLKSC
PHOSPHO1-3a GGDVAFPRRGYPMHRLIQEAQKAEPSSFRASVVPWETAADVRLHLQQVLKSC